Optimum pH and Temperature Conditions for Xylose Fermentation by *Pichia stipitis*

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Pichia stipitis NRRL Y-7124 is a xylose-fermenting yeast able to accumulate ca. 57 g/L ethanol. Because optimum process conditions are important, data were collected to determine the effects of temperature and pH on growth and fermentation rates and product accumulations. Temperatures (26–35°C) providing optimum biomass and ethanol productivities did not necessarily provide maximum ethanol accumulation. Xylitol and residual xylose concentrations increased with temperature. Maximum ethanol selectivity was achieved at 25–26°C with minimal sacrifice to production rates. The temperature optimum for xylose could not be generalized to glucose fermentations, in which ethanol productivity and accumulation were optimum at 34°C. The optimum pH range for growth and fermentation on xylose was 4–7 at 25°C.

INTRODUCTION

Pichia stipitis NRRL Y-7124 (CBS 5773) is an important xylose-fermenting yeast because it is able to accumulate enough ethanol (ca. 57 g/L) for economical recovery.[1] Temperature and pH requirements of this strain have been described by van Dijken and Scheffers who first identified the yeast's capacity to ferment xylose.[2] Their data indicated the temperature ranges for optimum initial growth and fermentation rates to be 28–32°C and 32–34°C, respectively, and the corresponding pH ranges to be 3–7 and 3–8, respectively. Other studies have shown that temperature was critical to controlling the relative concentrations of ethanol and xylitol produced by *Pachysolen tannophilus* NRRL Y-2460[3] and *Candida shehatae* CBS 2779.[4] In these yeasts, stalled xylose consumption and elevated xylitol byproduction become more problematic as temperature is increased. These findings suggest that other parameters, such as ethanol and xylitol accumulation, in addition to growth and fermentation rates, should be considered when choosing optimum process temperature and pH. This study examines the effects of temperature and pH on the concentration of products accumulated as well as on the rates of accumulation. Its results clarify important points about the pH and temperature needed to achieve a successful xylose fermentation by *Pichia stipitis* NRRL Y-7124.

MATERIALS AND METHODS

Organism and Media

Lyophilized *Pichia stipitis* NRRL Y-7124 (CBS 5773) was obtained from the ARS Culture Collection (Northern Regional Research Center, Peoria, IL). Stock cultures were maintained on agar slants incubated 48 h at 30°C and stored at 4°C. Fifty-milliliter liquid cultures adapted to growth on xylose (50 g/L) were the source of fermentor inocula. Adaptation to xylose involved a 24-h liquid culture inoculated from a slant and transferred (0.5 mL) twice more at 24-h intervals. These cultures were contained in 125-mL flasks with silicon sponge stoppers (Bellco) and were swirled with a 1-in. stroke at 150 rpm in a New Brunswick Psychrotherm at 30°C. Media used for slants (YM) and liquid cultures (CCY) were described previously.[5] CCY medium contained yeast extract, urea, potassium phosphate buffer, and various mineral salts, and in fermentors it was supplemented with 1 g/L Hodag FD-62 silicone antifoam.

Specific Growth Rate

Batch culture experiments to determine the dependence of specific growth rate on temperature and pH were carried out in B. Braun Biostat 2ER (2-L) fermentors. Vessels were equipped with temperature, pH, and dissolved oxygen control and were filled with CCY medium to a 1.5-L working volume. With pH the experimental variable, tem-

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perature was controlled at 30°C, and with temperature the variable, 6*N* NaOH was added to maintain pH at 4.5. In order to minimize changes in medium composition and prolong the logarithmic growth phase, cultures were inoculated to low initial cell concentrations of ca. 0.017 g/L (0.1 absorbance units). Growth rates were maximized by adding an excess but uninhibitory amount of xylose (40 g/L) to the media and by controlling oxygen concentration at 95% of saturation (assuming 902 mm Hg air pressure). Automatic oxygen control was accomplished by variable stirring (100–300 rpm) and air flow (0–12 L/min) rates. The culture absorbance (A) was monitored for about 12 h after inoculation, and specific growth rate [$\mu_{log} = d(\ln A)/dt$] was calculated by linear regression of ln(A) versus time to find the slope during logarithmic growth phase.

Fermentation Performance

Batch cultures with high concentrations of *P. stipitis* were monitored to assess the effects of temperature and pH on specific ethanol productivity and on ethanol and xylitol accumulations. Cells were produced in a New Brunswick Fermacel CF50 fermentor containing 20 L CCY medium with 150 g/L xylose and 1 g/L antifoam. Culture conditions were 30°C, 200 rpm stirring, and 3 L/min air flow rate. After 48 h, cells were harvested with a Sharpels continuous centrifuge. Enough cell paste was resuspended in Biostat or New Brunswick Microferm fermentors to bring the absorbance of the 2-L CCY volume to ca. 30–40 (5–7 g/L dry cells). Both fermentors were equipped with automatic foam control. The pH-controlled fermentations were done in Biostats with initial xylose at 150 g/L, temperature at 25 or 30°C, and oxygen transfer coefficient ($K_l a$) at 0.165 min^{-1}. Temperature effects were assessed in Microferms with initial xylose at 40 or 150 g/L (compared with glucose at 150 g/L), initial pH at 4.5 (final pH ca. 4), and $K_l a$ at 0.095 min^{-1}.

Specific ethanol productivity (p_E) was calculated from the initial (ca. 0–10 h) linear portion of the ethanol time course. Total biomass concentration was relatively constant over this period, and an average value ($b_{T,av}$) was obtained from absorbance data. Linear regression gave the volumetric productivity (P_E) as the slope of the ethanol time course, and p_E was evaluated as $P_E/b_{T,av}$.

Light Absorbance

Sample absorbance (A) was measured at 620 rn on a Bausch & Lomb Spectronic 2000 spectrophotometer and then used to calculate total dry biomass concentration (b_T). When necessary, samples were diluted such that $0.05 \leq A \leq 0.5$ units, where Beer's law is linear with concentration. For *P. stipitis*, $b_T = kA$ where $k = 0.167$ g/L.

Ethanol, Xylitol, Xylose, and Glucose Measurements

Samples were centrifuged, filtered through a 0.45-μm syringe filter, and stored at −20°C until analysis. Ethanol concentration (E) was determined with a Packard model 428 Gas Chromatograph equipped with a 6-ft Porapak Q column operating at 150°C. The D-xylose (X), D-glucose (G), and xylitol (XOH) concentrations were determined with a Waters high performance liquid chromatography (HPLC) system equipped with a Biorad HPX-87H Aminex ion exclusion column and a refractive index detector. The column was operated at room temperature, and acidified water (0.0017*N* H_2SO_4) was used as the mobile phase.

RESULTS AND DISCUSSION

Features of Growth and Fermentation Timecourses

Typical of our experiments initiated at $A = 0.1$ absorbance units, logarithmic cell growth was preceded by a lag and concluded by a stationary phase (time course data not shown). Both the lag time (2 h) and the stationary cell concentration were insensitive to changes in pH and temperature except under extreme conditions when growth was poor. Characteristic of our fermentations initiated at $A = 30$–40 absorbance units, ethanol accumulation passed through a maximum (E_{max}) as xylose concentration was depleted and ethanol consumption began. Xylitol was coproduced, but its final concentration (XOH_f) was stable with time, indicating that its consumption was negligible (Fig. 1). Residual xylose concentration (X_f), specific growth (μ_{log}) and fermentation (p_E) rates, and the accumulations of each product (E_{max} and XOH_f) were functions of pH and temperature.

Optima

Growth pH and Temperature

In the presence of 40 g/L xylose, μ_{log} was optimum (0.55–0.58 h^{-1}) over the pH range 4–7 and the temperature range 23–33°C (Figs. 2 and 3). As temperature in-

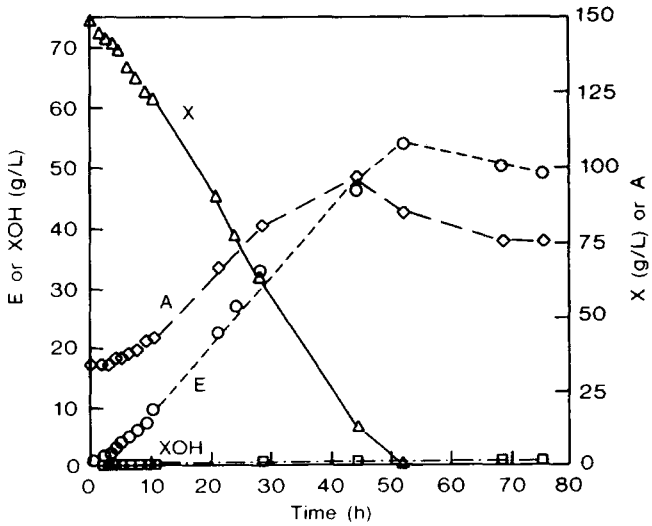

Figure 1. Time courses of culture absorbance (A) and concentrations of ethanol (E), xylitol (XOH), and xylose (X) during fermentation at 25°C, pH 5.0, and $K_l a = 0.165$ min^{-1}.

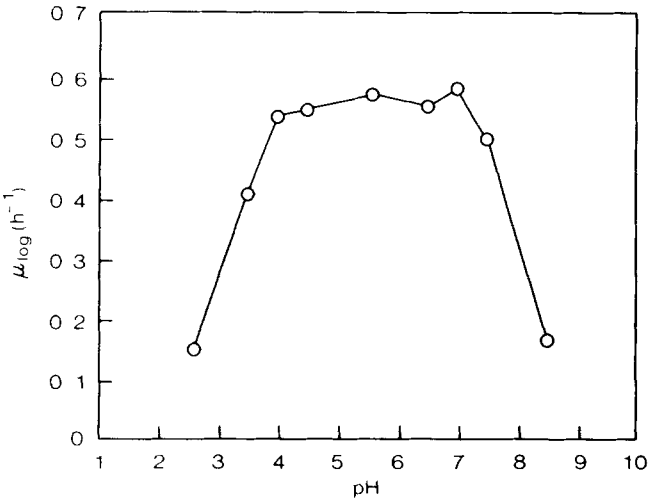

Figure 2. Dependence of specific growth rate (μ_{log}) on pH at 30°C, 40 g/L xylose, and 95% of dissolved oxygen saturation in cultures inoculated to 0.1 absorbance units.

creased, μ_{log} obeyed an Arrhenius dependence below 23°C, but it declined rapidly above 33°C. These optimum ranges for cell growth agree with those reported by van Dijken and Scheffers.[2] Our values of μ_{log} are 20% higher than those of van Dijken and Scheffers, but they agree with the values (ca. 0.55) reported by Delgenes and coworkers.[6] Comparison of experimental methods suggests that the higher growth rates may be attributed to more rapid air transfer and avoidance of oxygen limitation. The breadth of the optima observed for Y-7124 is not common to all strains of *P. stipitis*, however. For example, strain CSIR-Y633 (CBS-7126) had very narrow optima, pH 4–5.5 and 30°C, which applied to both growth and fermentation rates as well as ethanol yield on 50 g/L xylose.[4]

Fermentation pH and Temperature

Regardless of temperature (25 or 30°C), E_{max} and p_E were optimum over the broad pH range 4–7 (Fig. 4). Previously

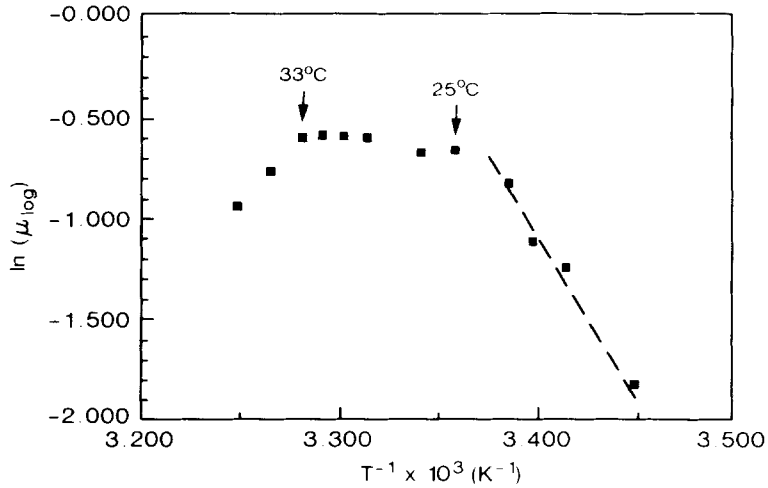

Figure 3. Arrhenius dependence of growth (μ_{log}) on temperature at pH 4.5, 40 g/L xylose, and 95% of dissolved oxygen saturation in cultures inoculated to 0.1 absorbance units. Linear regression fit of the equation, $\ln(\mu_{log}) = -(E_a/R)(1/T) + \ln(A_r)$, gave $A_r = 4.96 \times 10^{21}$ h^{-1} and $E_a/R = 15020$°K.

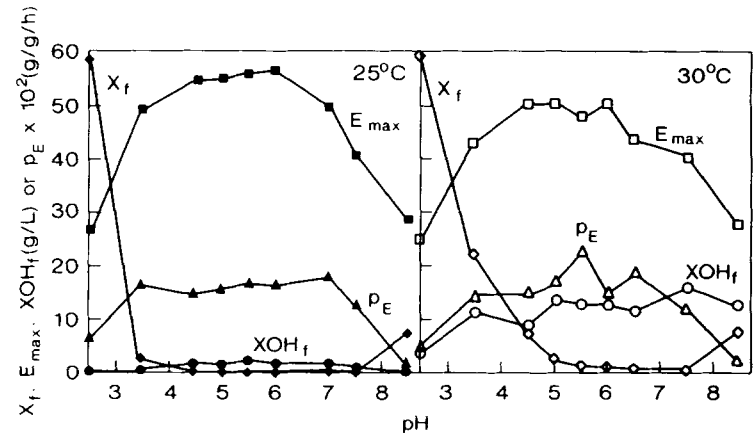

Figure 4. pH dependence of specific productivity (p_E) and final concentrations of ethanol (E_{max}), xylitol (XOH_f), and xylose (X_f) at 25 and 30°C (K_la = 0.165 min^{-1}; initial xylose = 150 g/L; initial absorbance = 30–40).

reported fermentation rates reflect a similar dependence on pH,[2] but the influences of pH and temperature on product accumulation and substrate utilization were not considered. Our experiments showed that E_{max} was higher at 25°C than at 30°C. The byproduct concentration XOH_f increased with pH but was lower at 25°C than at 30°C. Temperature also affected the pH range that allows complete sugar uptake. At 25°C, X_f was zero at pH 3.5–7.5, but at 30°C, X_f was near zero at pH 5–7.5.

Figure 5 shows that p_E and E_{max} were optimum (X_f and XOH_f minimum) at 23–30°C when initial xylose concentration (X_0) was 40 g/L. When X_0 was raised to 150 g/L, optimum p_E was shifted toward higher temperatures (27–33°C), but optimum E_{max} (minimum X_f and XOH_f) was achieved at <27°C [Fig. 3(a)]. The significance of temperature to ethanol and xylitol accumulations has been noted previously with respect to xylose fermentation by *Candida shehatae* (CBS 2779), *Pichia stipitis* (CSIR-Y633),[4,7] and *Pachysolen tannophilus* (NRRL Y-2460).[3] Growth studies on *P. stipitis* CSIR-Y633 suggest that ethanol tolerance decreases with increasing temperature.[7] This finding is consistent with our observations of *P. tannophilus* NRRL Y-2460 and *P. stipitis* NRRL Y-7124 having optimum ethanol accumulations ca. 25°C as opposed to 30–32°C, the production rate optimum. However, comparison of these reports and our data demonstrates that the effect of temperature on product selectivity varies considerably between xylose-fermenting strains. CSIR-Y633, for example, does not produce xylitol unless temperature is raised above 36°C, but CBS 2779, NRRL Y-2460, and Y-7124 produce it at as low as 25°C.[4] Thus, the choice of optimum process temperature is not universal among xylose-fermenting yeasts and must be based on characteristics of individual strains.

When 150 g/L glucose was the carbon source, E_{max} was optimum in the 25–34°C range, and p_E increased with temperature to an optimum of ca. 34°C. Figure 6 was included for comparison with Figure 5 to demonstrate a temperature effect that is unique to xylose metabolism and key to controlling the relative concentrations of ethanol and xylitol produced. However, it also teaches us that the choice of optimum process temperature will be significantly different for glucose and xylose, and cannot be generalized for all substrates.

CONCLUSIONS

The temperature of choice for xylose fermentation by *Pichia stipitis* NRRL Y-7124 was 25–26°C because it maximized E_{max}, minimized residual carbon as xylitol and xylose, and caused little sacrifice to μ_{log} and p_E. A greater sacrifice in p_E was called for at 150 g/L than at 40 g/L xylose. This choice of temperature could not be generalized to glucose fermentations, in which optimum p_E and E_{max} were achieved at 34°C. During xylose fermentation at 25°C, pH 4–7 allowed optimum μ_{log}, p_E, and E_{max}. Consistent with tradition, pH 4.5 is recommended for Y-7124 because it minimizes the likelihood of contamination. However, an automatic pH controller is desirable because 4.5 is near the lower limit for optimal fermentation.

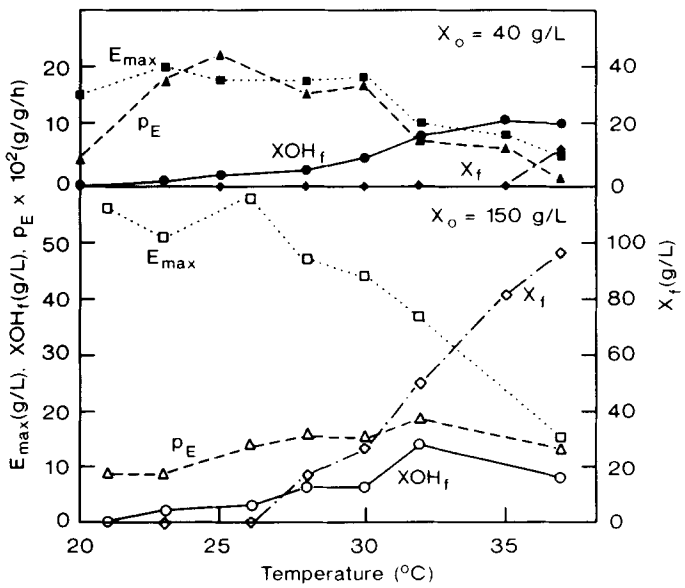

Figure 5. Temperature dependence of specific productivity (p_E) and final concentrations of ethanol (E_{max}), xylitol (XOH_f), and xylose (X_f) at initial xylose concentrations (X_0) of 40 and 50 g/L (pH 4.5–4.0; $K_l a$ = 0.095 min^{-1}; initial absorbance = 30–40).

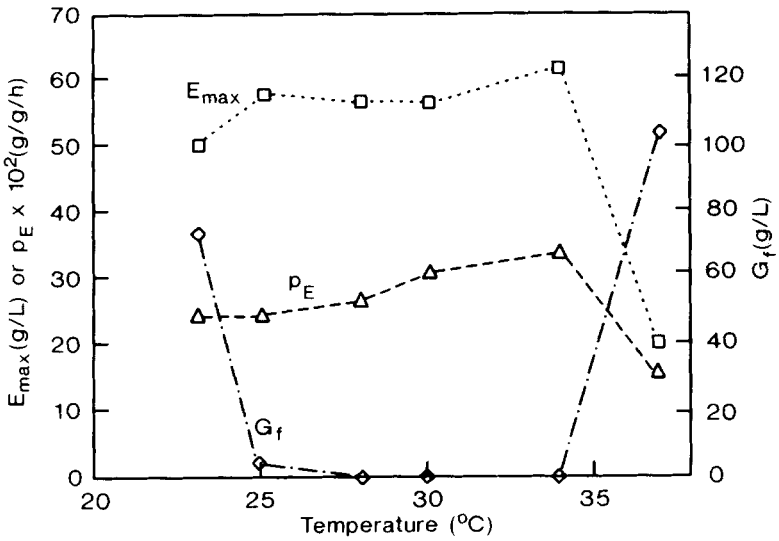

Figure 6. Temperature dependence of specific productivity (p_E) and final concentrations of ethanol (E_{max}) and glucose (G_f) (pH 4.5–4.0; K_la = 0.095 min^{-1}; initial glucose = 150 g/L; initial absorbance = 30–40).

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